by administering to a subject an antibody or protein that in and of itself, in the absence of conjugation to a cytostatic or cytotoxic agent, exerts a cytostatic or cytotoxic effect on Hodgkin's Disease cells. Support for the foregoing amendments can be found throughout the specification, *inter alia* at page 4, lines 3-8, at page 5, lines 33-36, and at page 52, lines 7-10. Claims 13-16 have been amended to remove dependencies upon canceled claims. No new matter is added.

THE REJECTION UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, FOR LACK OF ENABLEMENT, SHOULD BE WITHDRAWN

Claims 1-8, 11, and 13-19 are rejected under 35 U.S.C. § 112, first paragraph, allegedly because "the specification, while being enabling for the treatment of Hodgkin's disease, does not reasonably provide enablement for the prevention for Hodgkin's disease."

In no way acquiescing with the Examiner's rejection, and merely to expedite prosecution, Applicants have amended independent claims 1, 8 and 11 by deleting reference to prevention of Hodgkin's Disease. After entry of the amendments made herein, all pending claims are directed to the treatment of Hodgkin's Disease which, by the Examiner's own admission, is enabled.

Applicants respectfully submit that the rejection of the claims under 35 U.S.C. § 112, first paragraph, for lack of enablement, has been obviated and should be withdrawn.

THE REJECTION UNDER 35 U.S.C. § 102(b) SHOULD BE WITHDRAWN

The Examiner has rejected claims 1-5, 7, 8, 13, 15, 16 and 19 as being anticipated by U.S. Patent No. 5,165,923 to Thorpe ("Thorpe"). Applicants rsepectfully disagree. Thorpe does not anticipate the claimed invention, for the reasons discussed below.

First, Applicants note that claim 1 has been amended to clarify that claim 1 is directed to the treatment of Hodgkin's Disease using anti-CD30 antibodies and proteins that are themselves cytostatic or cytotoxic to Hodgkin's Disease cells in the absence of conjugation to a cytostatic or cytotoxic agent such as a chemotherapeutic agent or a toxin. In contrast, Thorpe teaches immunotoxins comprising a CD30 antibody or antibody derivative conjugated to a toxin moiety and the use of such immunotoxins to treat Hodgkin's Disease. However, unlike the antibodies and proteins of the present invention, the antibody portions of the immunotoxins disclosed by Thorpe have no inherent cell killing or growth inhibiting properties, unlike the antibodies employed in the methods of the present invention. This is

explicitly stated in Thorpe, for example at column 24, lines 57-60 (stating that the "cytotoxic effect of all the immunotoxins was specific [to the ricin moiety] since the native antibodies...were not toxic [to the Hodgkin's Disease cell line]). In direct contrast, the specification states that the antibodies and proteins of the present invention, such as AC10 and HeFi-1, are "distinguished from the previously described anti-CD30 mAbs by their [inherent] ability to inhibit the growth of CD30-expressing HD lines." (See the specification at page 52, lines 7-10).

With respect to claim 8 and claims dependent thereon, Applicants submit that Thorpe does not anticipate these claims, for the reason that Thorpe does not teach the killing or growth inhibition of Hodgkin's Disease cells using anti-CD30 an antibody that competes with AC10 or HeFi-1 for binding to CD30. In particular, Thorpe teaches treating Hodgkin's Disease using an antibody that cross-blocks by 70% the binding of HRS-3 to L540 cells when present at about a 100-fold excess with respect to HRS-3. In the experimental Section, Thorpe presents data regarding the ability of the antibodies HRS-1, HRS-3, HRS-4, Ber-H2, Ki-1, when conjugated to a toxin, namely the ricin A chain (see Example I, Section II.D at columns 24-25). Of these antibodies, HRS-1 and Ki-1, even when conjugated to the ricin A chain, were only weakly toxic to L540 cells (see column 24, lines 50-53). None of HRS-1, HRS-3, HRS-4, Ber-H2, Ki-1, however, compete with AC10 or HeFi-1 for binding to CD30. In support for the foregoing, Applicants invite the Examiner's attention to Horn-Lohrens *et al.*, 1995, "Shedding of the Soluble Form of CD30 from the Hodgkin-analogous Cell Line L540 Is Strongly Inhibited by a New CD30-specific Antibody (Ki-4)," Int. J. Cancer 60:539-544 ("Horn-Lohrens"), which is reference AS of record. In particular, the Examiner's attention is directed to the section on page 541 entitled "*Binding-inhibition experiments with anti-CD30 MAbs*," which describes the results of competitive inhibition studies among various anti-CD30 antibodies. The outcome of such competitive inhibition studies was a grouping of anti-CD30 antibodies into three groups based on cross-inhibition. The antibodies employed by Thorpe fell into Groups A (Ber-H2, HRS-1, and HRS-4) and B (Ki-1), and did not compete with AC10 or HeFi-1, which fell into Group C, for binding to CD30 (see the first paragraph on the right hand column of page 541 of Horn-Lohrens). Although Horn-Lohrens did not categorize the antibody HRS-3, the art indicates that, like HRS-4, HRS-3 also belongs to what Horn-Lohrens refers to as Group A anti-CD30 antibodies. As evidence of the foregoing, the Examiner's attention is directed to the Thorpe patent itself, in particular at column 23, lines 28 to 41, and Figure 1, which shows that HRS-3, HRS-4 and Ber-H2

effectively block each other's binding to CD30, placing HRS-3 in Horn-Lohrens' Group A. Additionally, Applicants direct the Examiner's attention to Hombach *et al.*, 1998, "Generation of the single chain antibody fragment conserves the idiotypic profile of the anti-CD30 monoclonal antibody HRS3," Scand. J. Immunol. 48(5):497-501 ("Hombach"), attached hereto as Exhibit C. The data presented in Figure 2 of Hombach confirm the finding of Thorpe that HRS-4 avidly competes with binding to CD30 with HRS-3. Based on the foregoing, one of skill in the art would conclude that the anti-CD30 antibodies HRS-3, as well as HRS-1, HRS-4, Ber-H2, and Ki-1, do not compete with AC10 or HeFi-1 for binding to CD30, and therefore, Thorpe does not anticipate the presently claimed invention of claim 8.

In view of the foregoing, Applicants submit that the rejection of claims 1 and 8, and claims 5, 7, 8, 13, 15, 16 and 19 dependent thereon, is in error and/or has been obviated by the present amendments and thus should be withdrawn.

THE REJECTION UNDER 35 U.S.C. § 103(a) SHOULD BE WITHDRAWN

Claims 1-8 and 13-19 are rejected under 35 U.S.C. § 103(a), allegedly as being obvious over Thorpe in view of Engert *et al.*, "Treatment of Advanced Hodgkin's Lymphoma: Standard and Experimental Approaches," Seminars in Hematology 36(3):282-289 ("Engert"). Applicants respectfully disagree for the reasons discussed below.

First, with respect to Thorpe, Applicants reiterate that Thorpe does not teach the use of an anti-CD30 antibody that is cytostatic or cytotoxic to Hodgkin's Disease cells independently of the toxin to which it is conjugated to treat Hodgkin's Disease, as is claimed in claim 1 and claims 2-7 dependent thereon. Further, Thorpe does not suggest the treatment of Hodgkin's Disease with an anti-CD30 antibody that in the absence of conjugation to a cytotoxic or cytostatic agent has cytotoxic or cytostatic properties against Hodgkin's Disease cells, nor does Thorpe provide any motivation to do so. Accordingly, Thorpe does not render obvious the invention of claims 1-7. With respect to claim 8 and claims 13-19 dependent thereon, Thorpe does not teach or suggest the use of a monoclonal antibody that competes with AC10 or HeFi-1 binding to CD30 for the treatment of Hodgkin's Disease, nor does Thorpe provide any motivation for doing so. Accordingly, Thorpe does not render obvious the invention of claims 8 and 13-19.

Engert does not remedy the deficiencies of Thorpe. Engert discusses the use of chemotherapy for treatment of Hodgkin's Disease. Engert further discusses the use of

immunotherapy, including immunotherapy with anti-CD30 antibody based immunotoxins, to treat Hodgkin's Disease. However, contrary to the Examiner's assertions, Engert does not suggest the use of anti-CD30 antibody-toxin conjugates in combination with chemotherapy. Moreover, Engert does not teach or suggest the use of an anti-CD30 antibody that is cytostatic or cytotoxic to Hodgkin's Disease cells independently of the toxin to which it is conjugated to treat Hodgkin's Disease, alone or in combination with immunotherapy. Further, Engert does not teach or suggest the use of a monoclonal antibody that competes with AC10 or HeFi-1 binding to CD30 for the treatment of Hodgkin's Disease, alone or in combination with chemotherapy. Yet further, Engert does not provide any motivation for the foregoing methods. Therefore, Engert, whether alone or in combination with Thorpe, does not render obvious the presently claimed invention.

Applicants submit that the rejection of claims 1-8 and 13-19 under 35 U.S.C. § 103(a) is in error and respectfully request that the rejection be withdrawn.

CONCLUSION

Applicants respectfully request that the above-made amendments and remarks be entered and made of record in the file history of the present application. In view of the amendments and remarks above, it is submitted that all the outstanding rejections have been overcome or obviated. Further, it is submitted that the claims are in form for allowance. If any issues remain, the Examiner is respectfully requested to telephone the undersigned at (212) 790-2247 to discuss any issues or questions.

Respectfully submitted,

Date: October 17, 2001

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Enclosures

Exhibit A
U.S. Application No. 09/724,406
Marked Up Version of Amended Claims

1. (Amended) A method for the treatment [or prevention] of Hodgkin's Disease in a subject comprising administering to the subject, in an amount effective for said treatment [or prevention], (a) an antibody that (i) immunospecifically binds CD30 and (ii) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, wherein said antibody exerts the cytostatic or cytotoxic effect on the Hodgkin's Disease cell line in the absence of conjugation to a cytostatic or cytotoxic agent, respectively; and (b) a pharmaceutically acceptable carrier.

7. (Amended) The method of claim 1, wherein the cytostatic or cytotoxic effect is [determined by] exhibited upon performing a method comprising:

(a) contacting a culture of the Hodgkin's Disease cell line with the antibody, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

(b) exposing the culture to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

(c) measuring the incorporation of ^{3}H-thymidine into cells of the culture,

wherein the antibody has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the antibody.

8. (Amended) A method for the treatment [or prevention] of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and (b) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, which amount is effective for the treatment [or prevention] of Hodgkin's Disease.

11. (Amended) A method for the treatment [or prevention] of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) comprises an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID

NO:10, and (b) immunospecifically binds CD30, which amount is effective for the treatment [or prevention] of Hodgkin's Disease.

13. (Amended) The method of any one of claims [8-12] 8 or 11, wherein the protein is a human, humanized or chimeric antibody.

14. (Amended) The method of any one of claims [8-12] 8 or 11, further comprising administering chemotherapy to said subject.

15. (Amended) The method of any one of claims [8-12] 8 or 11, wherein the protein is conjugated to a cytotoxic agent.

16. (Amended) The method of any one of claims [8-12] 8 or 11, wherein the protein is a fusion protein comprising the amino acid sequence of a second protein.

19. (Amended) The method of any one of claims [8-12] 8 or 11, wherein the cytostatic or cytotoxic effect is [determined by] exhibited upon performing a method comprising:

(a) contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

(b) exposing the culture to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

(c) measuring the incorporation of ^{3}H-thymidine into cells of the culture,

wherein the protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein.

Exhibit B
U.S. Application No. 09/724,406
Claims as Pending Following Entry of Amendments Made Herein

1. (Amended) A method for the treatment of Hodgkin's Disease in a subject comprising administering to the subject, in an amount effective for said treatment, (a) an antibody that (i) immunospecifically binds CD30 and (ii) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, wherein said antibody exerts the cytostatic or cytotoxic effect on the Hodgkin's Disease cell line in the absence of conjugation to a cytostatic or cytotoxic agent, respectively; and (b) a pharmaceutically acceptable carrier.

2. The method of claim 1, wherein the antibody is human, humanized or chimeric.

3. The method of claim 1, further comprising administering chemotherapy to said subject.

4. The method of claim 1, wherein the antibody is conjugated to a cytotoxic agent.

5. The method of claim 1, wherein the antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

6. The method of claim 4 or 5, further comprising administering chemotherapy to said subject.

7. (Amended) The method of claim 1, wherein the cytostatic or cytotoxic effect is exhibited upon performing a method comprising:

(a) contacting a culture of the Hodgkin's Disease cell line with the antibody, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

(b) exposing the culture to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

(c) measuring the incorporation of ^{3}H-thymidine into cells of the culture,

wherein the antibody has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the antibody.

8. (Amended) A method for the treatment of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and (b) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, which amount is effective for the treatment of Hodgkin's Disease.

11. (Amended) A method for the treatment of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) comprises an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:10, and (b) immunospecifically binds CD30, which amount is effective for the treatment of Hodgkin's Disease.

13. (Amended) The method of any one of claims 8 or 11, wherein the protein is a human, humanized or chimeric antibody.

14. (Amended) The method of any one of claims 8 or 11, further comprising administering chemotherapy to said subject.

15. (Amended) The method of any one of claims 8 or 11, wherein the protein is conjugated to a cytotoxic agent.

16. (Amended) The method of any one of claims 8 or 11, wherein the protein is a fusion protein comprising the amino acid sequence of a second protein.

17. The method of claim 15, further comprising administering chemotherapy to the subject.

18. The method of claim16, further comprising administering chemotherapy to the subject.

19. (Amended) The method of any one of claims 8 or 11, wherein the cytostatic or cytotoxic effect is exhibited upon performing a method comprising:

(a) contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

(b) exposing the culture to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

(c) measuring the incorporation of ^{3}H-thymidine into cells of the culture,

wherein the protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein.

nd. J. Immunol. 48, 497–501, 1998

Generation of the Single Chain Antibody Fragment Conserves the Idiotypic Profile of the Anti-CD30 Monoclonal Antibody HRS3

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Hombach A, Pohl C, Heuser C, Sircar R, Koch D, Diehl V, Abken H. Generation of the Single Chain Antibody Fragment Conserves the Idiotypic Profile of the Anti-CD30 Monoclonal Antibody HRS3. Scand J Immunol 1998;48:497–501

Recombinant single chain antibody fragments (scFv) derived by combining immunoglobulin VL and VH regions provide valuable antibody-like reagents. A number of them are shown to have retained the antigen specificity of the parental monoclonal antibody (MoAb). Little is known about the idiotypic profile of scFv fragments compared with that of the parental MoAb. To address this question we analysed the idiotypic profile of a scFv that was derived by phage-display techniques from the anti-CD30 MoAb HRS3. We assayed (i) binding of HRS3-scFv to recombinant CD30-Fc antigen and to four different anti-idiotypic MoAbs defining at least three different idiotopes on HRS3, and (ii) cross-competition with the parental MoAb HRS3 and the closely related anti-CD30 MoAb HRS4. The assays revealed that the HRS3-scFv fragment exhibits the same specificity for both CD30 antigen and the tested anti-idiotypic MoAbs compared with the parental MoAb demonstrating that the recombinant scFv fragment has retained the complete idiotope of the parental MoAb.

Hinrich Abken, Klinik I für Innere Medizin, Labor für Tumorgenetik, Universität zu Köln, Josef-Stelzmann-Str. 9, D-50924, Köln, Germany

INTRODUCTION

Single chain antibody fragments (scFv) are assembled by joining the variable region of the immunoglobulin (Ig) light (VL) and heavy (VH) chains via a short flexible peptide linker providing a powerful new tool in the recombinant antibody technology [1]. Isolation of scFv by phage-display techniques and expression of the scFv as fusion protein with the gp3 capsid antigen of a filamentious phage results in a recombinant phage antibody. Ig V region-specific cDNA derived from both libraries and hybridoma cells have been successfully assembled to scFv-phage antibodies with specificity to particular antigens [2, 3]. These scFv-type antigen-binding domains have stimulated increasing interest in recombinant antibody technology because of their potential for diagnostic or therapeutic purposes [4].

Although a number of scFv fragments retain the antigen specificity of the parental monoclonal antibody (MoAb), little is known about the idiotypic profile of these recombinant antibody-like fragments. Since scFv fragments consist entirely of the immunoglobulin VH- and VL-domains linked together in a single polypeptide chain their idiotypic profile may be different from that of the parental MoAb resulting in a loss of idiotopes and thus loss of reactivity with those anti-idiotypic MoAbs that define the respective idiotope. Whereas anti-idiotypic MoAbs are valuable tools for the generation, detection and purification of recombinant scFv fragments [5, 6], alterations in the idiotypic profile of the parental MoAb compared with the corresponding scFv fragment may result in differences in the reactivity pattern of the scFv fragment leading to cross-reactivity with unrelated antigens. A retained idiotypic profile will be of particular importance when the scFv antibody fragment, alone or as part of fusion proteins, is used *in vivo* for diagnostic or therapeutic purposes.

To address this question, we analysed the idiotypic profile of a recombinant anti-CD30-scFv utilizing a panel of anti-idiotypic MoAbs. The anti-CD30-scFv was derived from the MoAb HRS3 by phage-display techniques and found to exhibit the same specificity as the parental MoAb with respect to the CD30 antigen. We utilized anti-idiotypic MoAbs with specificity for the anti-CD30 MoAb HRS3 to display the idiotypic profile of the recombinant HRS3-scFv. Highly expressed on Hodgkin's lymphoma cells [7] CD30 is successfully used as a target for a

panel of anti-CD30 antibodies in the diagnosis and immunotherapy of Hodgkin's lymphoma [8–10]. An anti-CD30 scFv with retained anti-idiotypic profile would therefore be of particular interest.

MATERIALS AND METHODS

Cell lines and antibodies. The hybridoma cell lines HRS3 and HRS4 produce the MoAbs HRS3 (IgG1) and HRS4 (IgG1), respectively, with specificity to the CD30 antigen [9, 11, 12]. The hybridoma cell lines producing the anti-idiotypic MoAbs 9G10, 14G9 and 14B12 (all IgG1) were derived by immunization with the anti-CD30 MoAb HRS4. The hybridoma line producing the anti-idiotypic MoAb 12D3 (IgG1) was derived by immunization with the anti-CD30 MoAb HRS3 [11, 12]. All cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS). The MoAbs were purified from cell culture supernatants by affinity chromatography utilizing antimouse IgG agarose (Sigma, Deisenhofen, Germany). The recombinant CD30–human immunoglobulin Fc fusion protein [13] and the mouse CC49–scFv human IgG Fc fusion protein with specificity for the tumour antigen TAG72 [14] were purified from supernatants of transfected CHO and myeloma cells, respectively, by affinity chromatography utilizing anti-human IgG agarose (Sigma).

Cloning and isolation of the HRS3-single chain antibody fragment (HRS3-scFv). The VH- and VL-immunoglobulin cDNA sequences of the MoAb HRS3 were amplified using PCR techniques by means of immunoglobulin-specific primers (Pharmacia, Freiburg, Germany), and assembled by PCR techniques via a linker coding for a $(Gly_4\ Ser)_3$ peptide. The assembled product (750 bp) was reamplified by means of oligonucleotides introducing *Sfi*I and *Not*I restriction sites, respectively, and ligated into the *Sfi*I and *Not*I sites of pCANTAB 5E vector DNA (Pharmacia), hereby inserting the scFv DNA in frame with the E-Tag DNA and the M13 gene 3 DNA. Recombinant phages were expressed by phage-display techniques and enriched by panning using the anti-idiotype MoAb 9G10. Specific binding of HRS3-scFv-M13 phages to MoAb 9G10 was assayed by ELISA.

Detection of the recombinant phage antibodies. Microtitre plates were coated with anti-idiotypic antibodies and an IgG1 control MoAb (2 μg/ml each), respectively, or with CD30–Fc and CC49-scFv–Fc fusion protein (2 μg/ml each), respectively. After incubation overnight at 4°C, the plates were blocked and incubated for 1 h with supernatants containing HRS3-scFv phage antibody. Bound phage antibodies were detected by a peroxidase-conjugated anti-M13 antibody (1:5000) (Pharmacia). The titre of the recombinant phages was determined by ELISA utilizing an immobilized anti-M13 antibody (Pharmacia). For control reasons, the binding studies were also performed in parallel with the phage antibody B72.3-scFv that exhibits specificity for the tumour antigen TAG72 and bovine submaxillary mucin (BSM) [15]. Bound phage were detected as described above and standardized to a defined titre of M13KO7 phages (Pharmacia).

Cross-competition of HRS3-scFv. Microtitre plates were coated with anti-idiotypic antibodies (2 μg/ml each) or with CD30–Fc fusion protein (2 μg/ml). After incubation overnight at 4°C, the plates were blocked and incubated for 1 h with HRS3-scFv phage antibody containing supernatants in the presence of different amounts (0.001–10 μg/ml) of the parental anti-CD30 MoAb HRS3, the anti-CD30 MoAb HRS4, or an isotype-matched IgG1 control MoAb. Bound phage antibodies were detected as described above. The binding inhibition (%) was determined as follows:

100 × (binding without competition)/(binding with competition).

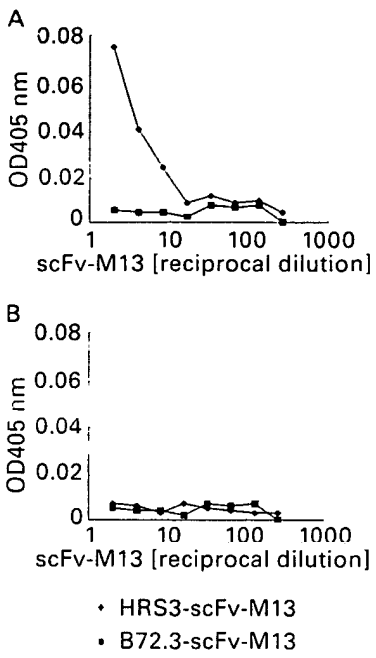

Fig. 1. Specific binding of HRS3-scFv to CD30-Fc antigen. Reciprocal dilutions of HRS3-scFv-M13 and B72.3-scFv-M13 phages (1×10^{10} pfu/ml each), respectively, were incubated in microtitre plates coated with 2 μg/ml of either CD30-Fc (A) or CC49-Fc recombinant antigen (B). Bound phage antibodies were detected by an anti-M13-antibody conjugated with peroxidase (1:5000). The phage titre was standardized by ELISA as described in Materials and methods.

RESULTS

Binding of HRS3-scFv to recombinant CD30 fusion protein

We generated the HRS3-single chain antibody fragment (HRS3-scFv) using phage-display techniques joining the immunoglobulin VH- and VL-cDNA sequences derived from the HRS3

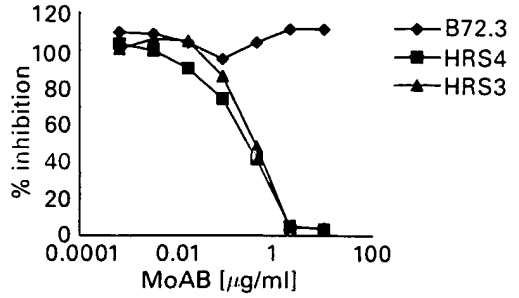

Fig. 2. Binding inhibition of HRS3-scFv to CD30-Fc antigen. HRS3-scFv-M13 phages (5×10^9 pfu/ml) were incubated in microtitre plates coated with 2 μg/ml CD30-Fc antigen protein in the presence of increasing amounts of the anti-CD30 MoAbs HRS3, HRS4, and an isotype matched control MoAb (B72.3), respectively. Bound phage antibodies were detected by an anti-M13 antibody conjugated with peroxidase (1:5000) and the inhibition (%) was calculated.

Table 1. Characteristics of anti-idiotypic (anti-id) MoAbs

	Immunogen* reactivity†		Ab3			
anti-id MoAb	HRS3	HRS4	HRS3	HRS4	(anti-CD30)‡	DTH§
9G10	−	+	+	+	+	+
14G9	−	+	+	+	+	+
14B12	−	+	+	+	−	−
12D3	+	−	+	−	−	−

*Four different anti-idiotypic MoAbs were obtained after immunization of BALB/c mice with either MoAb HRS3 or HRS4 [11, 12].

†Reactivity of MoAb HRS3 and HRS4, respectively, with the anti-idiotypic MoAbs [11, 12].

‡BALB/c mice and rabbits, respectively, were immunized with anti-idiotypic MoAbs. Polyclonal antibodies of immunized animals were analysed for binding to CD30 antigen by flow cytometry and Western blot [11, 12].

§BALB/c mice were immunized with anti-idiotypic MoAbs and delayed-type hypersensitivity reactin (DTH) was tested by injection of CD30+ lymphoma cells into the footpad of immunized animals. DTH-reaction was significant for animals upon immunization with MoAb 9G10 ($P<0.001$) and 14G9 ($P<0.01$) [11, 12]

hybridoma cells as described in Materials and Methods. The HRS3-scFv domain is part of the HRS3-scFv–gp3 fusion protein that is expressed on the surface of M13 phage. A number of recombinant phage clones were screened for binding to the anti-idiotypic MoAb 9G10. One clone with specific binding to MoAb 9G10 was isolated, and the DNA sequence determined and analysed in detail.

To prove the antigen-binding specificity of the isolated HRS3-scFv recombinant CD30 human IgG Fc fusion protein was immobilized and incubated with HRS3-scFv- and B72.3-scFv phage supernatants, respectively, containing the same number of M13 phage. HRS3-scFv was found to bind to CD30-Fc antigen whereas the B72.3-scFv with specificity to TAG72 antigen and BSM did not (Fig. 1). No reactivity of HRS3-scFv against an unrelated Fc fusion antigen (CC49-Fc) was observed demonstrating that HRS3-scFv has retained specificity for the CD30 antigen. Binding of HRS3-scFv to CD30-Fc antigen was inhibited by both the parental MoAb HRS3 and, moreover, the anti-CD30 MoAb HRS4 revealing a closely related epitope on the CD30 antigen for both MoAbs (Fig. 2). An isotope-matched IgG1 control MoAb did not inhibit specific binding.

Binding of HRS3-scFv to anti-idiotypic MoAbs

We analysed the idiotope of the recombinant HRS3-scFv antibody fragment with four different anti-idiotypic MoAbs which define at least three different idiotopes. The properties of the utilized MoAbs are summarized in Table 1. The MoAbs 9G10, 14G9 and 14B12 were obtained by immunization of mice with the anti-CD30 MoAb HRS4 [11, 12] and were found to react with

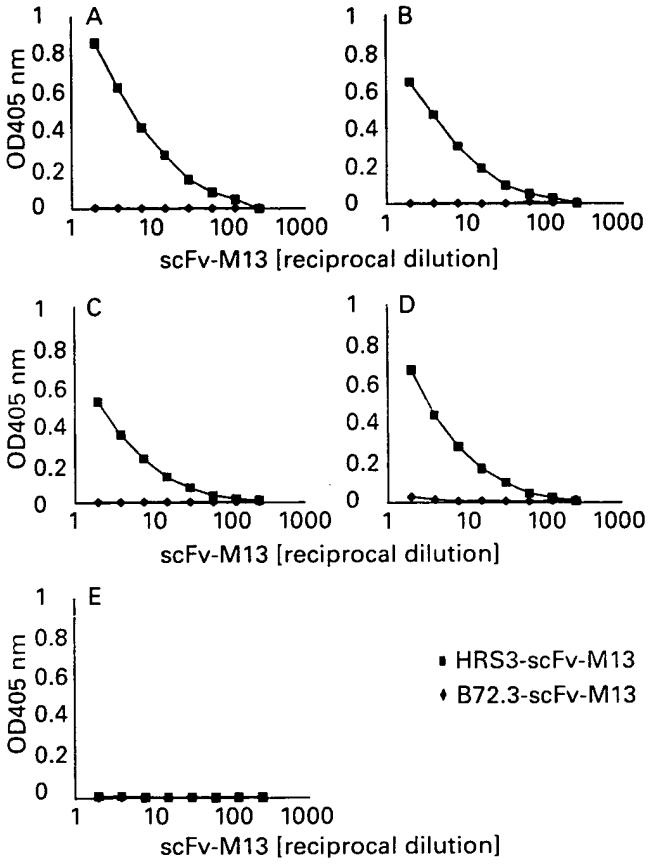

Fig. 3. Specific binding of HRS3-scFv to different anti-HRS3 idiotypic MoAbs. Reciprocal dilutions of HRS3-scFv-M13 and B72.3-scFv-M13 phages (1×10^{10} pfu/ml each), respectively, were incubated in microtitre plates coated with 2 μg/ml of the anti-idiotypic MoAbs 9G10, 14G9, 14B12, and 12D3 (A–D) and an isotype matched control MoAb (E). Bound phage antibodies were detected by an anti-M13 antibody conjugated with peroxidase (1:5000). The phage titre was standardized by ELISA.

both MoAb HRS4 and the closely related MoAb HRS3. Moreover, the MoAbs 9G10 and 14G9 proved to carry an internal image of the CD30 antigen. The MoAb 12D3 was obtained after immunization with MoAb HRS3 (unpublished data). In contrast to the other antibodies, MoAb 12D3 reacts exclusively with the anti-CD30 MoAb HRS3.

Immobilized anti-idiotypic MoAbs were assayed with HRS3-scFv- and with B72.3-scFv phage supernatants, respectively, containing same numbers of M13 phage. As demonstrated in Fig. 3, HRS3-scFv reacted specifically with the four anti-idiotypic MoAbs tested, whereas the B72.3-scFv used as a control did not. The idiotypic binding pattern of HRS3-scFv is identical to that of the parental MoAb HRS3.

Specific binding of HRS3-scFv to the anti-HRS4 and anti-HRS3 idiotypic MoAbs, respectively, was assayed by binding-inhibition experiments utilizing the parental anti-CD30 MoAb HRS3 and the closely related anti-CD30 MoAb HRS4, respectively. Binding of HRS3-scFv to the anti-idiotypic MoAbs was

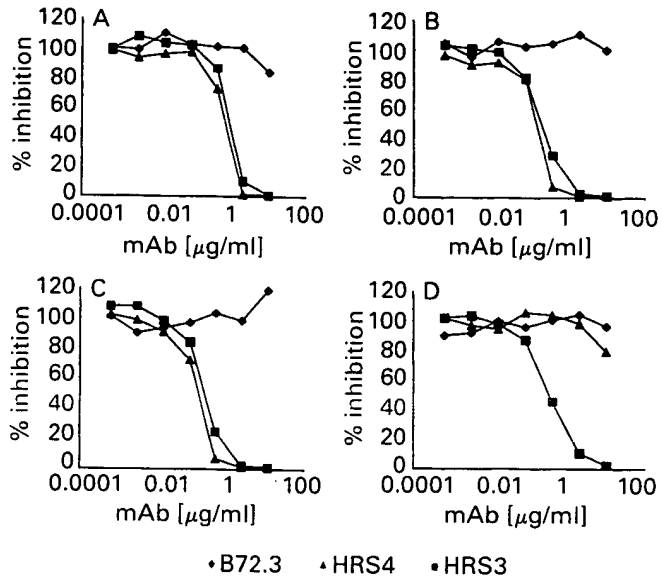

Fig. 4. Inhibition of binding of HRS3-scFv to anti-idiotypic MoAbs. HRS3-scFv-M13 phages (5×10^9 pfu/ml) were incubated in microtitre plates coated with 2 μg/ml anti-idiotypic MoAbs in the presence of increasing amounts of the anti-CD30 MoAbs HRS3, HRS4 or an isotype matched control MoAb (B72.3). Bound phage antibodies were detected by an anti-M13 antibody peroxidase conjugated (1:5000) and the inhibition [%] was calculated.

inhibited by the parental MoAb HRS3 whereas an isotope-matched IgG1 control MoAb did not (Fig. 4). In contrast, the anti-CD30 MoAb HRS4 inhibited binding of HRS3-scFv to the anti-idiotypic MoAbs 9G10, 14G9 and 14B12 (Fig. 4A–C) that were obtained after immunization with MoAb HRS4. Co-incubation with MoAb HRS4 did not inhibit binding of HRS3-scFv to the anti-idiotypic MoAb 12D3 (Fig. 4D). The same cross-competition pattern was observed by utilizing the parental MoAb HRS3.

Taken together, the binding pattern and the specific cross-competition demonstrate that the recombinant HRS3-scFv antibody fragment has retained the idiotypic profile of the parental MoAb HRS3.

DISCUSSION

Single chain antibody fragments (scFv) are known to retain the specificity of the parental MoAb from which they are derived [15–17], whereas the idiotypic profile of these scFv, to our knowledge, has not to date been compared with that of the parental MoAb. Here we investigated the idiotypic profile of a recombinant anti-CD30-scFv antibody fragment utilizing four different anti-idiotypic MoAbs that define at least three different idiotopes on the anti-CD30 MoAb HRS3 (Table 1) [11, 12]. Two anti-idiotypic MoAbs (9G10, 14G9) were shown to carry an internal image of the CD30 antigen, whereas the other anti-idiotypic MoAbs (14B12, 12D3) define two additional distinct idiotopes of MoAb HRS3. Binding-inhibition and cross-reactivity studies demonstrate that the scFv fragment has retained the complete idiotypic profile of the parental MoAb, as defined by the four different anti-idiotypic MoAbs. Studies on single chain antibody fragments, however, derived from different MoAbs indicate that scFv antibody fragments do not always retain the complete idiotypic profile (U. Reinhold, unpublished observation). Accordingly, we and others isolated scFv fragments that have lost some idiotypic determinants resulting in anti-idiotypic reactivity patterns that are different to those of the parental MoAb although these scFv antibody fragments have retained binding to the particular antigen. Loss of idiotypic determinants of the recombinant scFv antibody fragment, however, may result in different reactivity patterns compared with the parental MoAb. The anti-CD30 MoAbs HRS3 and HRS4 are closely related with respect to binding specificity for a closely related epitope of the CD30 antigen as demonstrated by cross-competition assays on CD30+ lymphoma cells [9]. Accordingly, upon immunization with MoAb HRS4, several anti-idiotypic MoAbs were obtained that bind to MoAb HRS3 as well. Despite the high similarity in binding to CD30 antigen and anti-idiotypic MoAb, both MoAbs exhibit significant differences in their binding pattern to the anti-idiotypic MoAb which was obtained after immunization with MoAb HRS3 and CD30-negative tissues. Whereas the specificity of MoAb HRS3 is restricted to CD30+ cells, MoAb HRS4 shows cross-reactivity to CD30-pancreas cells [9] demonstrating that even minor differences in the idiotope of an antibody may result in different binding patterns on particular tissues. For this reason, the idiotypic profile of a recombinant scFv fragment is considered to be of particular interest. Based on our results exclusively those scFv antibody fragments that have retained the complete idiotype of the parental MoAb are expected to exhibit the reactivity pattern of the parental MoAb. Manipulation of the binding sites, e.g. by humanization of scFv either by grafting murine CDR regions into the murine framework regions or by chain shuffling utilizing human Ig VH- and VL-libraries, may be accompanied by alterations in the idiotopic profile. However, interest in recombinant anti-CD30 antibodies with a defined idiotypic profile increases because a panel of CD30-specific antibodies is available that is successfully used in the diagnosis and immunotherapy of Hodgkin's lymphoma [8–10]. The recombinant HRS3-scFv retaining the idiotypic profile of the parental MoAb HRS3 will therefore be a valuable and highly specific tool for targeting CD30+ Hodgkin's lymphoma cells and, moreover, may also prove to be useful in the development of a recombinant anti-idiotypic vaccine that is expected to be as efficient as the MoAb HRS3.

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